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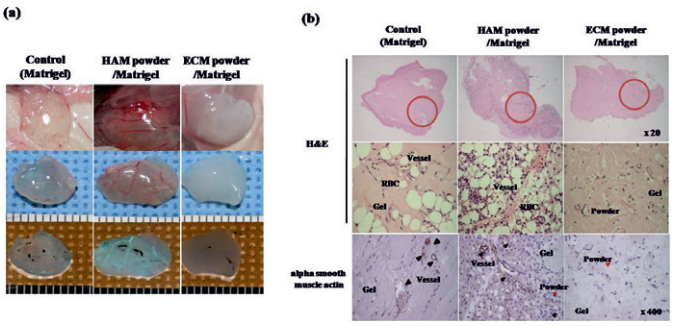

Fig. 2. Gross observation and histology of retrieved samples from the *in vivo* experiment. (a) The implanted specimens (n = 5/time point/group) were retrieved at 1 week after implantation. The gross images of specimen were presented vessel invasion *in vivo*. (b) The sections were stained with hematoxylin/eosin and alpha smooth muscle actin to observed vessel invasion. Scale bar: 1 mm for 20× and 200 μm for 400× image.

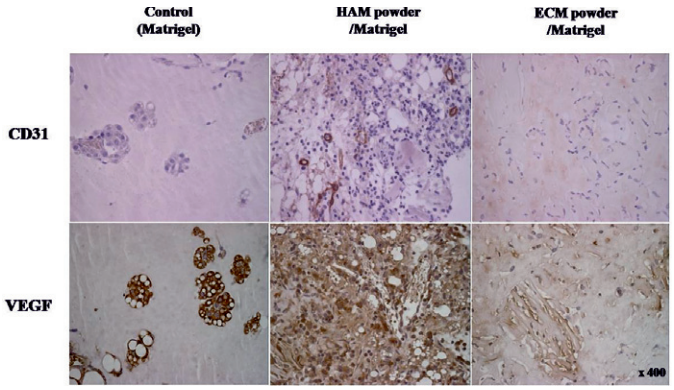

Fig. 3. Immunostaining of in vivo specimens for CD31 and VEGF-A. The retrieved specimens at 1 week were immunostained CD31, VEGF-A to observed vessel invasion. Scale bar: 200 μm for 400× image.

Tube formations of HUVECs were significantly reduced on a basement membrane matrix containing the ECM powder in their vicinity than basement membrane matrix containing the HAM powder (Fig. 1b). In the nude mice model, the vessel invasion also occurred more deeply and intensively in Matrigel containing HAM than in the one containing CD-ECM (Fig. 2a). Also, express of alpha smooth muscle actin was observed in the broad central area, similar to H&E stain in Matrigel containing HAM. However, Matrigel containing CD-ECM was not observed vessel-like structures (Fig. 2b). In the immunostaining the expression of CD31, an endothelial cell marker was not expressed in the ECM powder/Matrigel, while expressed in the whole region of the HAM powder/Matrigel. The expression of VEGF-A also showed a positive stain in the whole region of the HAM powder/Matrigel samples

Conclusion: These results suggest that the extracellular matrix material from chondrocytes has potential to inhibit vessel invasion *in vitro* and *in vivo*.

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LYSYL HYDROXYLASE 2B IS STRONGLY INDUCED DURING EXPERIMENTAL OA AND THE POTENTIAL CAUSE OF PERSISTENT SYNOVIAL FIBROSIS

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Purpose: Fibrosis is a major contributor to stiffness in osteoarthritis (OA) and associated with joint pain. Lysyl hydroxylase 2b (LH2b) increases pyridinoline cross-links during collagen synthesis. These cross-links are harder to degrade than "normal" cross-links and are elevated during different fibrotic diseases. In previous studies we found a relationship between LH2b and irreversible fibrosis after TGF-β exposure in murine knee joints. Furthermore, we showed that TGF-β induced LH2b gene expression in human synovial OA fibroblasts (hSF). In this study we examined whether LH2b is actually induced in experimental OA. Thereafter we investigated which TGF-β signaling route is involved in up regulation of LH2b expression and whether gene expression also translates into LH2 protein production by TGF-β in hSF.

Methods: To induce OA we injected of bacterial collagenase into the right knee joint of C57BL/6 mice. The mice were sacrificed at day 7, 21 and 42 after collagenase injection and the mRNA was isolated from the synovium for Q-PCR analysis. Human synovium fibroblast were isolated from knee joints of OA patients undergoing arthroplasties. The hSF were transduced with adenoviruses overexpressing TGF-β (Ad-TGF-β), CTGF (Ad-CTGF) or Luc (Ad-Luc) to determine whether the LH2b induction is a TGF-β specific effect, as found in mice. Thereafter, the hSF were stimulated with TGF-β with and without ALK5 or ALK1 kinase inhibitor, SB-505124 (SB) or dorsomorphin (DM) respectively. In addition, cells were transduced with constitutive active TGF-β type I receptors, Ad-caALK1 or Ad-caALK5 to determine whether LH2b expression is ALK1 or ALK5 specific. RNA was isolated and the gene expression for LH1, -2 and -3, lysyl oxidase (LOX), collagen type 1A1 (COL 1A1), and CTGF was analyzed with RT-PCR. LH2 protein level was determined with Western Blot analysis.

Results: In murine experimental OA there was a significant and long-lasting increase of LH2b gene expression in the synovium on day 7, 21 and 42 compared to the control knee joints (figure 1A). In hSF Ad-TGF-β induced LH2b gene expression whereas Ad-CTGF did not, comparable to our observations in murine synovium. TGF-β stimulation induced LH2b, CTGF, COL1A1 and LOX gene expression as well as LH2 protein (figure 1B).

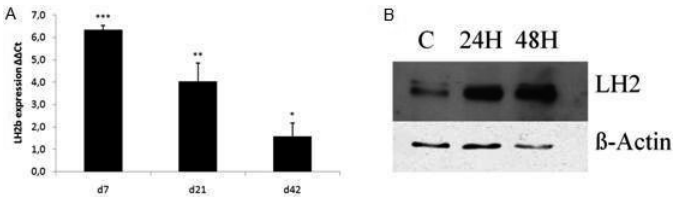

Fig. 1. (A) LH2b gene expression measured in mRNA isolated from the synovium of murine knee joints with collagenese induced OA. The LH2b gene expression was significantly induced in the OA-affected joint compared to the healthy joints on al measured days (day 7, 21 and 42). (B) Human synovial fibroblasts were cultured in the presence or absence of TGF-β for 24 h and 48 h. A strong increase in LH2 protein level was observed after both 24h and 48h TGF-β stimulation.

No major changes in LH1 and LH3 gene expression were found. Ad-caALK5 but not Ad-caALK1 significantly induced LH2b. This was confirmed by SB completely blocking TGF-β induced LH2b, while LH2b gene expression was only slightly decreased by DM. Identical results were observed for all five primary fibroblast cell cultures.

Conclusions: In this study we have shown that LH2b is strongly induced in an experimental OA model that is accompanied by synovial fibrosis. Furthermore, we showed that both LH2b gene expression and LH2 protein are induced by TGF-β in hSF. Overexpressing caALK5, the canonical TGF-β type I receptor, induced LH2b, whereas blocking ALK5 kinase activity prevented TGF-β induced LH2b. The overexpression of caALK1 did not alter LH2b gene expression, indicating TGF-β-induced LH2b relies on ALK5 signaling alone. During OA, TGF-β is elevated causing enhanced LH2b expression. LH2b increases pyridinoline cross-links in collagen. We therefore propose that LH2b will be responsible for the persistence of fibrosis during OA. Selective blocking of LH2b in OA may prevent the formation of the pyridinoline cross-links, and therefore the formation of persistent fibrosis. Thus LH2b is a highly interesting new target to treat OA-related fibrosis.

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EFFECT OF REMOBILIZATION ON IMMOBILIZATION-INDUCED JOINT CONTRACTURE IN RAT KNEE JOINTS

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Purpose: Joint immobilization is commonly used for the treatment of many musculoskeletal disorders, but it also causes joint contracture. Recent studies suggested that a joint capsule, which belongs to arthrogenic components, is a main cause of joint contracture. In our previous reports, immobilization induced restriction in range of

motion after 4 weeks in a rat model, which might be caused by adhesion and fibrosis of the synovial membrane and increased stiffness of the capsule. Restoration of joint contracture after remobilization is almost unknown. The purpose of this study is to clarify effects of remobilization on immobilization-induced joint contracture by histological and histomorphometric approaches, and the measurement of the joint angle.

Methods: *Experimental design*: Eighty and sixty four adult male Sprague-Dawley rats aged 12 weeks were prepared for histology and measurement of the joint angle. Unilateral knee joints were immobilized at 150° of flexion with a plastic plate and metal screws for 1, 2, 4, 8, and 16 weeks (n = 8/each period). For the joint angle, we measured from 1 to 8 weeks. Only screws were inserted for sham-operated rats. After the immobilization periods, the fixation devices were removed and the rats were allowed to move freely in standard cages for 16 weeks. The immobilized rats and sham-operated rats made up the immobilized-remobilized (Im-Re) group and the control group, respectively.

Histology & immunocytochemistry: The rats were fixed with 4% PFA in 0.1M PBS at a 150° flexed position, and the resected knee joints were decalcified in 10% EDTA in 0.01M PBS and embedded in paraffin. Five-μm sections were obtained at the medial mid-condylar region of the knee in the sagittal plane. The sections were stained with Elastica-Masson and morphological changes were observed. The length of the superficial layer of the synovial membrane in the anterior and posterior capsule was measured. Distribution of type A (macrophage-like) synoviocytes was observed by the immunostaining of CD68 (MCA341R, Serotec, Oxford, U.K.).

Measurement of joint angle: The hindlimb of the rats was resected and the muscles were removed. The angle between the longitudinal axis of the femur and the tibia was measured under x-ray control. Two different torques (Torque 1: 225 g-cm, Torque 2: 450 g-cm) were chosen to extend the knee joint. After incision of the posterior capsule, the angle was measured again at two different torques (Torque 2 and Torque 3: 1350 g-cm).

Results: *Histology & immunocytochemistry*: Joint space was filled with fibrous tissues and shortening of the synovial membrane in the posterior capsule was observed in the 2-week Im-Re group and it developed after 4-week Im-Re groups. The length in the anterior capsule was significantly shorter in the 4-week Im-Re group. CD68 positive cells were observed in the superficial layer of the synovial membrane in the control group, but they were not observed in the Im-Re group.

Measurement of joint angle: Significant limitation in extension was observed in the 4 and 8-week Im-Re groups at torques 1 and 2. After incision of the posterior capsule, significant limitation was observed in the 8-week Im-Re group at torque 3.

Conclusions: The histological study indicated that synovial adhesion formed by joint immobilization was not restored by 16-week remobilization. Synoviocytes were not observed in the fibrous tissues formed by synovial adhesion, which might indicate that joint immobilization induced irreversible capsular changes. The measurement of the joint angle study showed that 4-week immobilization induced irreversible joint contracture caused by arthrogenic components. After incision of the posterior capsule, loss of the joint angle in the 4-week Im-Re group was restored, but it was not restored in the 8-week Im-Re group, which indicated that not only the joint capsule but also the other arthrogenic components such as the collateral and cruciate ligaments could also cause joint contracture.

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MEMBRANE-BOUND COMPLEMENT REGULATORY PROTEIN EXPRESSION DECREASES IN SYNOVIUM DURING OA PROGRESSION AND IS ASSOCIATED WITH INCREASED C5B-9 DEPOSITION

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Purpose: Though not considered a classical inflammatory arthropathy, osteoarthritis (OA) is often associated with low-grade synovitis. The stimuli necessary to establish inflammation in OA synovium are as yet unclear but there is emerging evidence to suggest complement pathway activation may have a role in this process. Studies have shown that advanced-stage OA synovium and cartilage produce complement proteins and that they are abundant in synovial fluid. In addition, several reports have also identified the membrane attack complex (MAC) of complement (C5b-9) in advanced-stage OA synovium and synovial fluid. Due to the potentially destructive nature of complement activation, the majority of host cells express membrane-bound complement regulatory proteins (CRP) to prevent damage. The aim of this study is to characterize the expression of the membrane-bound CRP CD46, CD55 and CD59 in synovium during OA progression as well as the effects of exposure to C5b-9 in relevant synovial cell populations.

Methods: Synovial biopsies were obtained from patients undergoing arthroscopic surgery due to anterior cruciate ligament (ACL) or degenerative meniscal tear and arthroplasty due to OA or rheumatoid arthritis (RA). Patients having arthroscopic surgery due to a degenerative meniscal tear were considered to have early OA (E.OA) if radiographic analysis resulted in a Kellgren-Lawrence (K-L) score of ≤2. In contrast, patients undergoing arthroplasty were considered to have advanced OA (A.OA) if the K-L score was >2. Patients having ACL tears generally showed no radiographic signs of cartilage wear. Membrane-bound CRP expression in synovial biopsies was determined by real time PCR and immunohistochemistry. C5b-9 deposition was analyzed in corresponding tissues and synovial fluid samples where possible by immunohistochemistry and ELISA respectively. Fibroblast-like synoviocytes (FLS) derived from A.OA synovium were stimulated with a sub-lytic 0.6 μg/mL concentration of C5b-9 over 72 hours and the expression of inflammatory cytokines/mediators and matrix-degrading enzymes was analyzed by real time PCR.

Results: Real time PCR analysis identified significantly decreased expression of *CD46* and *CD59* in synovium from A.OA and RA patients compared to those having ACL tear or E.OA. Reduced staining for CD59 was also observed in corresponding A.OA and RA tissue sections with more intense staining observed in the vasculature and intima of ACL tear and E.OA synovium. C5b-9 deposits were most prevalent in A.OA and RA tissues having an inverse relationship with CD59 expression. Despite differences in C5b-9 deposition observed in tissues, no differences were observed between C5b-9 concentrations in ACL tear, E.OA and A.OA synovial fluids, but levels were significantly higher in RA synovial fluids. OA FLS treated with sub-lytic C5b-9 exhibited significantly upregulated expression of the inflammatory mediator cyclooxygenase-2 (*COX2*) as well as matrix metalloproteinase-1 (*MMP1*).

Gene expression analysis of the membrane-bound CRP CD46, CD55 and CD59 in synovium (fold change)

Comparison	*CD46*	*CD55*	*CD59*
ACL vs. E.OA	0.8, $P < 0.01$	1.1, $P > 0.05$	1.0, $P > 0.05$
ACL vs. A.OA	1.4, $P < 0.01$	1.3, $P > 0.05$	1.2, $P < 0.05$
ACL vs. RA	1.7, $P < 0.001$	3.4, $P < 0.01$	1.6, $P < 0.01$
E.OA vs. A.OA	1.6, $P < 0.001$	1.1, $P > 0.05$	1.2, $P < 0.05$
E.OA vs. RA	2.1, $P < 0.001$	3.0, $P < 0.01$	1.6, $P < 0.01$
A.OA vs RA	1.3, $P > 0.05$	2.7, $P < 0.01$	1.3, $P > 0.05$

Conclusions: The reduced expression of CD46 and CD59 observed in A.OA may ultimately prevent adequate regulation of complement activation, an idea further supported by the increased deposition of C5b-9 observed in these tissues. These findings suggest uncontrolled complement activation may be one mechanism which contributes to the development of synovitis. This is supported by our experiments in OA FLS which demonstrate C5b-9 deposition can stimulate the gene expression of inflammatory mediators and matrix-degrading enzymes known to be associated with OA progression. A better understanding of the mechanisms which regulate membrane-bound CRP expression may be of value in developing strategies to prevent complement-associated tissue damage in OA. Furthermore, our studies suggest existing therapeutic strategies designed to inhibit complement activation may be of benefit to OA patients.